# Use of the Polymerase Chain Reaction to Investigate the Dynamics of Pyrethroid Resistance in *Haematobia irritans irritans* (Diptera: Muscidae)

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**ABSTRACT** A field study was conducted from 1991 through 1997 to evaluate the use of pyrethroid and organophosphate (OP) ear tags, alternated yearly, for the control of a pyrethroid resistant horn fly, *Haematobia irritans* (L.), population in Louisiana. Fly resistance was monitored by weekly fly counts, filter paper bioassays and diagnostic polymerase chain reaction (PCR) assays for the presence of pyrethroid resistance-associated mutations in the sodium channel gene coding region. Fly control in the first study year was poor, as pyrethroid ear tags were effective for only 7 wk. The following year, OP ear tags provided 15 wk of fly control. However, in all subsequent years, fly control was poor with both types of ear tags. The PCR assays showed that there were very few female flies homozygous for the pyrethroid susceptible sodium channel allele, never rising above 10% of the total females in the population. A fitness cost appeared to be associated with the pyrethroid resistant allele, as the resistant form was selected against in the absence of the pyrethroid ear tags. Despite this selection in favor of the susceptible allele and the annual alternation of pyrethroid and OP ear tags, the percentage of homozygous susceptible flies never reached over 19% of the population, resistant alleles of the sodium channel remained at high levels in the population, and horn fly control on cattle with either type of tag quickly became minimal.

**KEY WORDS** *Haematobia irritans*, polymerase chain reaction, resistance mechanism, overwintering, sodium channel, mutation

CONTROL OF THE horn fly, *Haematobia irritans* (L.), has become more problematic for cattle producers as this hematophagous cattle pest has become resistant to many of the chemical insecticides used for control. Resistance to organophosphates (OP) was first reported in 1963 (Burns and Wilson 1963). Subsequently there were reports of pyrethroid resistance in Florida and Louisiana and product failure in several locations in the United States (Kunz and Schmidt 1985, Quisenberry et al. 1984, Schmidt et al. 1985). Sparks et al. (1985) reviewed the status of insecticide resistance in the horn fly and noted several pyrethroid resistant populations in the southern United States. Later, pyrethroid resistance was reported in New Mexico (Crosby et al. 1991), Kentucky, (Cilek and Knapp 1993), and Manitoba (Mwangala and Galloway 1993). There are several mechanisms by which horn flies can develop resistance to pyrethroids (Sparks et al. 1985). However, McDonald and Schmidt (1990) concluded that one major target site-insensitivity gene was responsible for the rapid evolution of pyrethroid resistance from California to Florida.

The target site of pyrethroids is the sodium channel which is a transmembrane voltage-gated protein responsible for the generation of conducted electrical signals in neurons (Reviewed by Catterall 1995). Target site pyrethroid resistance has been documented in many insects, and particularly well-studied in *Drosophila melanogaster* (Meigan) (Pittendrigh et al. 1997) and *Musca domestica* L. (Williamson et al. 1996a). Single nucleotide substitutions in the S6 transmembrane segment of domain II (*kdr*) and the S4-S5 loop of domain II (*super-kdr*) of the sodium channel gene have been directly linked to target site pyrethroid insensitivity by electrophysiological studies comparing wild type and mutated house fly sodium channels expressed in *Xenopus* oocytes (Smith et al. 1997; Lee et al. 1999). As tools for resistance diagnosis, polymerase chain reaction (PCR) assays have been developed to detect these resistance-causing nucleotide substitutions in several species, including *M. domestica* (Williamson et al. 1996b) and *H. irritans* (Guerrero et al. 1998).

If there are no fitness costs associated with the *kdr* or *super-kdr* mutated sodium channel alleles, then

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once these resistance alleles become fixed in a population of horn flies, susceptibility might be difficult to recover. The refractory nature of target site pyrethroid resistance in the horn fly has been documented by the laboratory and field studies of resistant horn fly populations in New Mexico and Louisiana (Byford et al. 1999; Barros et al. 1999). After complete fly control failure using pyrethroid-containing ear tags, two consecutive years of treatment with OP-containing ear tags restored only partial pyrethroid susceptibility. The primary objective of the present research was to follow changes in allele frequency of the *kdr* and *super-kdr* pyrethroid resistance alleles in a pyrethroid resistant population of horn flies in Louisiana as treatment alternated yearly between synergized pyrethroid- and OP-containing ear tags. Filter paper bioassays (Sheppard and Hinkle 1987), fly counts, and diagnostic PCR resistance assays (Guerrero et al. 1998) were used to document changes in fly control, resistance to pyrethroid and OP ear tags, and *kdr* and *super-kdr* allele frequencies.

## Materials and Methods

**Horn Flies.** The study was conducted at the St. Joseph location of the Northeast Research Station, Louisiana Agricultural Center; fly collection and bioassay procedures were described in Barros et al. (1999). Weekly fly counts were conducted from at least 1 wk before ear tags were used until the end of the treatment period, or until loss of control. Loss of control was defined as fly counts averaging over 50 flies/side/animal. The total number of flies on one side of 10 randomly selected adult cows per site was estimated before 0830 hours (cdst) with the aid of binoculars. Insecticide susceptibility was determined by filter paper bioassay (Sheppard and Hinkle 1987) conducted once before the annual fly control treatment regime began and a second time after treatment. The insecticides used in these bioassays were diazinon (87.5% purity, Fermenta Animal Health, Kansas City, MO) and λ-cyhalothrin (93% purity, Mallinckrodt Veterinary, Mundelein, IL). Fly mortality was determined after a 4 h exposure period with flies unable to walk considered dead. Two-fold dilutions of insecticide were used in the range of 0.030-13.76 $\mu g/cm^2$ and 0.036-37.2 $\mu g/cm^2$ for diazinon and λ-cyhalothrin, respectively. Three replicates were used for each insecticide concentration and bioassay data analyzed by probit analysis using POLO-PC (LeOra Software 1987). Flies were collected by sweeping with hand nets before treatment and at least one week after ear tags were removed. A portion of the flies was immediately used for bioassays performed at the research farm location and the remainder frozen and stored at −80°C for use in PCR genotyping. Horn flies obtained from the Knipling-Bushland U.S. Livestock Insects Research Laboratory, USDA-ARS, Kerrville, TX, were used as an insecticide susceptible strain in the bioassays. The Kerrville fly bioassays were performed at the same time as the bioassays of the field-collected flies. Resistance ratios (R factors) were calculated by dividing the $LC_{50}$ for the tested population by the $LC_{50}$ for the Kerrville colony. The $LD_{50}$ data from the Kerrville reference strain varied between 0.019–0.079 $\mu g/cm^2$ and 0.32–0.46 $\mu g/cm^2$ for the cyhalothrin and diazinon bioassays, respectively.

Cattle were treated with two ear tags each on a yearly rotation between tags with pyrethroid plus synergist (Saber Extra, containing 10% cyhalothrin + 13% piperonyl butoxide) used in 1991, 1993, 1995, and 1997 and tags with an OP (Tomahawk, Dominator, and Rotator, containing 20% pirimiphos-methyl) used in 1992, 1994, and 1996. Except for 1997, the animals were also treated with 1% permethrin + 1% PBO pour-on (Synergized DeLice) at the time of tagging. The number of cattle ranged from 50–65 and were crossbred Angus (37.5–50%) X Hereford (25–50%), *Bos taurus* x Brahman (12.5–25%), *Bos indicus.* All insecticide-containing products were provided by Schering-Plough Animal Health (Union, NJ).

**Nucleic Acids.** Genomic DNA was isolated from individual frozen adult flies following a protocol reported by Guerrero et al. (2001) by using a disposable pellet pestle (Kontes, Vineland, NJ) to pulverize each fly in an individual 1.5-ml microcentrifuge tube prechilled on dry ice. Twenty-five microliters of sample buffer (100 mM Tris, pH 8.3; 500 mM KCl) was added to the tube and grinding continued for ≈20 s. The tube was transferred back to dry ice until a convenient number of flies was prepared. The tube was briefly microcentrifuged to ensure the liquid and crushed material were at the tube bottom, then placed in a boiling water bath for 3 min. After a 5-min centrifugation at 15,000 × *g*, an aliquot was diluted 1:10 in water and 2.5 μl of DNA solution was used for PCR.

PCR was performed by the procedure of Jamroz et al. (1998) in thin-walled microcentrifuge tubes (Bio-Rad, Hercules, CA) by using 20-μl reactions optimized for primer annealing temperature and sequence and $MgCl_2$ concentration to allow for the consistent discrimination between the susceptible and resistant sodium channel *kdr* and *super-kdr* alleles. Final optimized reaction conditions used 2.5 μl of diluted genomic DNA solution from a single fly (100–150 ng), primers as described below, 14 mM Tris (hydroxymethyl)aminomethane hydrochloride pH = 8.3, 70 mM KCl, 0.15 mM each dNTP, 4.5 mM $MgCl_2$, and 0.3 μl of a 1:1 vol:vol mix of *AmpliTaq* DNA Polymerase (5 U/μl stock; Perkin-Elmer, Foster City, CA) and TaqStart Antibody (1.1 μg/μl stock; Clontech, Palo Alto, CA). Primers were used at the following concentrations: R1, R2, R3, FG130, and FG134 = 1.5 μM; R5 and R6 = 1.75 μM; and R4 = 0.3 μM (Table 1). Each complete PCR pyrethroid resistance genotyping assay requires two amplification reactions. To assay for the presence of susceptible alleles, only primers R1–5 and FG130 were included in the reaction mix. To assay for resistant alleles, only primers R1–4, R6, and FG134 were included in the reaction mix. Amplification was carried out using a DNA Engine (MJ Research, Watertown, MA) programmed for 96°C for 2 min followed by 40 cycles, each consisting of denaturation at 94°C for 50 s, annealing at 62°C for 1 min, and extension

**Table 1. Sequences of PCR primers**

| Primer ID | Sequence | Description | Annealing site[a] |
|---|---|---|---|
| FG-130 | 5′-TACTGTTGTCATCGGCAATC-3′ | Sus forward *kdr* diagnostic[b] | nt #429–448 |
| FG-134 | 5′-TACTGTTGTCATCGGCAATT-3′ | Res forward *kdr* diagnostic[c] | nt #429–448 |
| R1 | 5′-GACAAATTCAAAGATCATGAAT-3′ | Forward control primer | nt #274–295 |
| R2 | 5′-TACGTTTCACCCAGTTCTTA-3′ | Reverse *kdr* control primer | nt #573–592 |
| R3 | 5′-TCGTGTATTCAAATTGGCAAA-3′ | Forward *super-kdr* control primer | nt #105–125 |
| R4 | 5′-CGAAAAGTTGCATTCCCATA-3′ | Reverse control primer | nt #231–250 |
| R5 | 5′-ACCCATTGTCCGGCCCA-3′ | Sus reverse *super-kdr* diagnostic[b] | nt #161–177 |
| R6 | 5′-ACCCATTGTCCGGCCCG-3′ | Res reverse *super-kdr* diagnostic[c] | nt #161–177 |

[a] Numbering is 5′-3′ on sense strand of horn fly sodium channel fragment (Guerrero et al. 1997).
[b] Susceptible horn fly sodium channel cDNA fragment Genbank Acc. no. U83871: nt #161–177 5′-TGGGCCGGACAATGGGT-3′, nt #429–448 5′-TACTGTTGTCATCGGCAATC-3′.
[c] Resistant horn fly sodium channel cDNA fragment Genbank Acc. no. U83873: nt #161–177 5′-CGGGCCGGACAATGGGT-3′, nt #429–448 5′-TACTGTTGTCATCGGCAATT-3′.

at 68°C for 1 min. The program also included a final extension step at 68°C for 7 min. Reaction products were fractionated on 3.5% NuSieve agarose (FMC BioProducts, Rockland, ME) TBE gels and DNA was visualized by staining with GelStar DNA Staining Dye (FMC Bioproducts, Rockland, ME) and UV illumination.

## Results

**Fly Control.** When the study was initiated in 1991, only 7 wk of horn fly control was achieved with pyrethroid ear tags (Table 2). Thus, even at the beginning of the study, horn flies at St. Joseph had a moderate level of resistance to pyrethroids. Subsequently, the level of control by pyrethroid ear tags slowly dropped even though pyrethroid ear tags were used only in alternate years; essentially no control occurred in 1997. The population's resistance factors for cyhalothrin in 1993, 1995, and 1997 before applying the cyhalothrin ear tags were 21, 150 and 22, while the number of weeks of control were 6, 4, and 2, respectively. The initial OP ear tag treatment in 1992 controlled horn flies for 15 wk, subsequently fly control dropped to four and 3 wk in 1994 and 1996, respectively. The resistance factors for diazinon in 1992, 1994 and 1996 were 1.0, 1, and 0.4, respectively, before applying the OP tags in the spring of those years. Therefore, neither the resistance factor nor $LD_{50}$ was predictive for the level of control attained by either the OP or the pyrethroid ear tags.

**Fly Genotyping.** Approximately 40 male and 40 female flies collected before and after each treatment were assayed by PCR to obtain a profile of the horn fly population at the *superkdr* and *kdr* alleles (Table 3). Mutated (R) *superkdr* alleles were never found in the absence of a mutated (R) *kdr* allele, thus the SR-SS, RR-SS and RR-SR (*superkdr-kdr*) genotypes are not represented in Table 3.

Regardless of the time of collection, the minority of tested flies (particularly females) in this population were susceptible (Fig. 1). The highest percentage (10%) of *kdr* susceptible female flies (SS) occurred in spring before the 1996 OP tagging. Because the *superkdr* resistance allele apparently does not occur in the absence of a preexisting *kdr* resistance allele (Jam-

**Table 2. Fly control and cyhalothrin and diazinon $LD_{50}$ data from cattle undergoing yearly ear tag rotation**

| Year | Tag[a] | Time[b] | λ-cyhalothrin | | Diazinon | | Control[d] (weeks) |
|---|---|---|---|---|---|---|---|
| | | | $LD_{50}$ (95% FL) μg/cm$^2$ | R factor[c] | $LD_{50}$ (95% FL) μg/cm$^2$ | R factor | |
| 1991 | PS | Pre | 1.9 (1.6–2.4) | — | 1.1 (0.74–1.4) | — | 7 |
| | | Post | — | — | — | — | |
| 1992 | OP | Pre | 5.8 (4.8–6.7) | 310 | 0.43 (0.36–0.49) | 1.0 | 15 |
| | | Post | 1.7 (−)[e] | 93 | — | — | |
| 1993 | PS | Pre | 1.0 (0.67–1.3) | 21 | 0.05 (0.03–0.08) | 0.1 | 6 |
| | | Post | 7.5 (5.5–11) | 160 | 0.55 (0.48–0.65) | 1.2 | |
| 1994 | OP | Pre | 5.7 (4.0–7.8) | 72 | 0.45 (0.37–0.54) | 1.0 | 4 |
| | | Post | — | — | — | — | |
| 1995 | PS | Pre | 3.4 (1.2–15) | 150 | 0.39 (−)[e] | 1.0 | 4 |
| | | Post | 9.9 (−)[e] | 440 | 0.64 (−)[e] | 1.6 | |
| 1996 | OP | Pre | — | 22 | 0.13 (0.11–0.15) | 0.4 | 3 |
| | | Post | — | 30 | 0.47 (0.34–0.63) | 1.3 | |
| 1997 | PS | Pre | 0.54 (0.16–1.2) | 22 | — | — | 2 |
| | | Post | 43 (25–240) | 1740 | 1.2 (−)[e] | 2.8 | |

[a] PS, pyrethroid + synergist; OP, organophosphate.
[b] Pre, bioassayed before tagging; post, bioassayed at least one week after tags removed.
[c] R factor, resistance factor = test $LD_{50}$ ÷ Kerrville reference susceptible colony $LD_{50}$.
[d] Defined as time until >50 flies/side/animal.
[e] Data too heterogeneous to calculate 95% fiducial limits.

**Table 3. *Superkdr-kdr* genotype of horn fly populations from cattle undergoing yearly ear tag rotation**

| Year | Tag[a] | Time[b] | Control[c] (weeks) | Sex | Total no. | *superkdr-kdr* genotype[d] (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | SS-SS | SS-SR | SS-RR | SR-SR | SR-RR | RR-RR |
| 1991 | PS | Pre | 7 | M | 41 | 24 | 66 | 10 | 0 | 0 | 0 |
| | | | | F | 40 | 8 | 52 | 40 | 0 | 0 | 0 |
| | | | | M + F | | 16 | 59 | 25 | 0 | 0 | 0 |
| | | Post | | M | 28 | 0 | 11 | 54 | 4 | 29 | 4 |
| | | | | F | 41 | 0 | 0 | 85 | 0 | 15 | 0 |
| | | | | M + F | | 0 | 4 | 72 | 1 | 20 | 1 |
| 1992 | OP | Pre | 15 | M | 42 | 2 | 48 | 45 | 0 | 5 | 0 |
| | | | | F | 32 | 0 | 16 | 81 | 0 | 3 | 0 |
| | | | | M + F | | 1 | 34 | 61 | 0 | 4 | 0 |
| | | Post | | M | 17 | 12 | 53 | 35 | 0 | 0 | 0 |
| | | | | F | 28 | 7 | 4 | 86 | 0 | 4 | 0 |
| | | | | M + F | | 9 | 22 | 67 | 0 | 2 | 0 |
| 1993 | PS | Pre | 6 | M | 38 | 8 | 50 | 39 | 0 | 3 | 0 |
| | | | | F | 41 | 7 | 34 | 59 | 0 | 0 | 0 |
| | | | | M + F | | 8 | 42 | 49 | 0 | 1 | 0 |
| | | Post | | M | 37 | 0 | 16 | 54 | 3 | 24 | 3 |
| | | | | F | 39 | 3 | 3 | 87 | 5 | 3 | 0 |
| | | | | M + F | | 1 | 9 | 71 | 4 | 13 | 1 |
| 1994 | OP | Pre | 4 | M | 42 | 2 | 48 | 33 | 2 | 14 | 0 |
| | | | | F | 39 | 0 | 26 | 72 | 0 | 3 | 0 |
| | | | | M + F | | 1 | 37 | 52 | 1 | 9 | 0 |
| | | Post | | M | 42 | 17 | 48 | 24 | 2 | 10 | 0 |
| | | | | F | 32 | 3 | 25 | 56 | 0 | 16 | 0 |
| | | | | M + F | | 11 | 38 | 38 | 1 | 12 | 0 |
| 1995 | PS | Pre | 4 | M | 41 | 24 | 66 | 5 | 0 | 2 | 2 |
| | | | | F | 36 | 6 | 53 | 33 | 3 | 6 | 0 |
| | | | | M + F | | 16 | 60 | 18 | 1 | 4 | 1 |
| | | Post | | M | 41 | 2 | 32 | 41 | 2 | 22 | 0 |
| | | | | F | 41 | 0 | 10 | 73 | 0 | 15 | 2 |
| | | | | M + F | | 1 | 21 | 57 | 1 | 18 | 1 |
| 1996 | OP | Pre | 3 | M | 42 | 19 | 60 | 12 | 10 | 0 | 0 |
| | | | | F | 42 | 10 | 45 | 38 | 5 | 2 | 0 |
| | | | | M + F | | 14 | 52 | 25 | 7 | 1 | 0 |
| | | Post | | M | 41 | 10 | 39 | 39 | 2 | 10 | 0 |
| | | | | F | 52 | 6 | 31 | 54 | 6 | 4 | 0 |
| | | | | M + F | | 8 | 34 | 47 | 4 | 6 | 0 |
| 1997 | PS | Pre | 2 | M | 32 | 34 | 53 | 9 | 3 | 0 | 0 |
| | | | | F | 41 | 7 | 61 | 24 | 2 | 5 | 0 |
| | | | | M + F | | 19 | 58 | 18 | 3 | 3 | 0 |
| | | Post | | M | 42 | 0 | 14 | 50 | 2 | 31 | 2 |
| | | | | F | 42 | 0 | 0 | 71 | 2 | 24 | 2 |
| | | | | M + F | | 0 | 7 | 61 | 2 | 27 | 2 |

[a] PS, pyrethroid + synergist; OP, organophosphate.
[b] Pre, bioassayed before tagging; post, bioassayed at least one week after tags removed.
[c] Defined as time until counted >50 flies/side/animal.
[d] Percentages of total where S denotes a susceptible allele, R denotes a resistant allele with *superkdr* genotype indicated on the left of hyphen and *kdr* genotype on right.

roz et al. 1998; Soderlund and Knipple 1999), flies which are SS for *kdr* must be SS for *superkdr*. During the 7-yr study, at five timepoints no SS females were detected. These were in the fall after the pyrethroid ear tag treatments in 1991, 1995, and 1997 and in the spring before the OP ear taggings in 1992 and 1994. In fact, except for fall of 1994 and 1996, over 90% of the female flies collected posttreatment had RR *kdr* alleles (Table 3). The only pretreatment spring samples that showed <40% RR *kdr* female flies were in 1995 and 1997. The males contained lower percentages of resistant alleles than the females. There were three samples that failed to detect any male SS flies. Each of these timepoints followed a pyrethroid ear tag treatment (1991, 1993, and 1997). Unlike the female samples, none of the male posttreatment fall samples contained >90% RR *kdr* flies; although values of 87, 81, and 83% RR flies were found in the fall samples of 1991, 1993, and 1997, respectively. Four samples of males contained relatively low numbers of RR *kdr* flies, these were prepyrethroid treatment samples obtained in 1991, 1995, 1997, and one preOP treatment sample in 1996.

The percentage of SS flies data (Fig. 1) gives the percentage of flies that would be expected to be easily killed by insecticide. The percentage of S alleles (Fig. 2) would include contributions from all susceptible flies (doubly weighted because they possess two S alleles) plus heterozygotes (singly weighted because they only possess one S allele). Fig. 2 shows the wide fluctuations in the *kdr* S allele frequency, especially evident after the pyrethroid ear taggings during 1991, 1993, 1995, and 1997. In general, large declines in percentage of S occurred after a summer during which

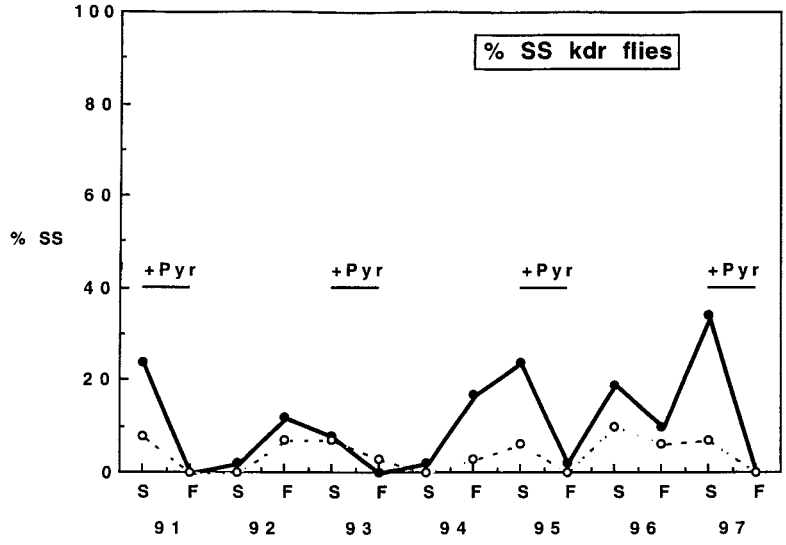


**Fig. 1.** Changes in pre- and posttreatment percentages of susceptible male and female horn flies. Approximately 40 male and 40 female flies were genotyped from collections in the spring (S) before and in the fall (F) after ear tagging during 1991–1997. Percentages of SS represents the percentage of the male (●) and female (○) flies whose two copies of the sodium channel gene had the susceptible genotype at the *kdr* allele. The times during which the pyrethroid ear tags were in use are indicated (+*Pyr*).

pyrethroid ear tags were used and an increase in percentage of S occurred after the winter after a summer during which pyrethroid ear tags were used.

The analysis of the *superkdr* alleles revealed that most flies were not homozygous resistant for *superkdr* (Table 3). Only two samples of females contained flies which were homozygous resistant (RR) at the *superkdr* allele, the postpyrethroid tagging samples from 1995 and 1997 each of that had only 2% *superkdr* RR flies. More than 80% of the female flies possessed the homozygous susceptible (SS) *superkdr* genotype except for the flies in the 1997 postpyrethroid treatment

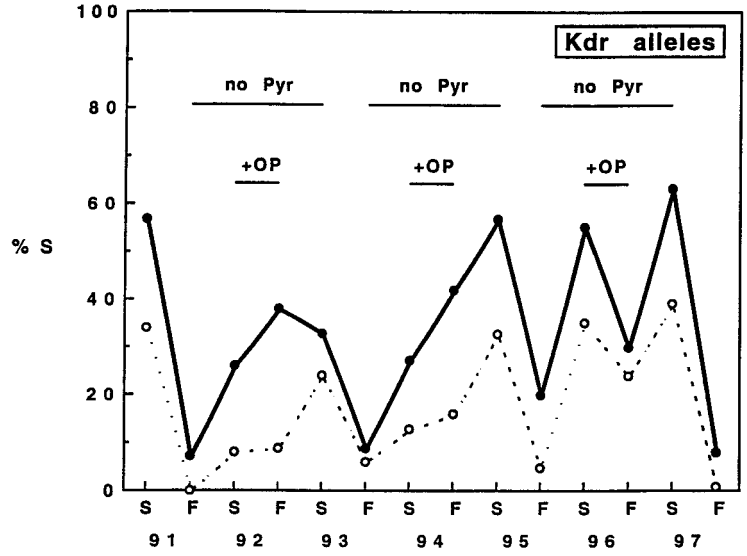


**Fig. 2.** Changes in pre- and posttreatment susceptible *kdr* allele frequencies in male and female horn flies. Approximately 40 male and 40 female flies were genotyped from collections in the spring (S) before and in the fall (F) after ear tagging during 1991–1997. Percentage of S represents the percentage of sodium channel alleles in males (●) and females (○) that have the susceptible *kdr* genotype. Percentages of S = [([number of SS flies] × 2 + number of SR flies) ÷ 2 × total number of flies assayed] × 100%. The times during which the OP ear tags were in use (+OP) are indicated, whereas the complete interval during which pyrethroids were not used (no Pyr) are indicated.

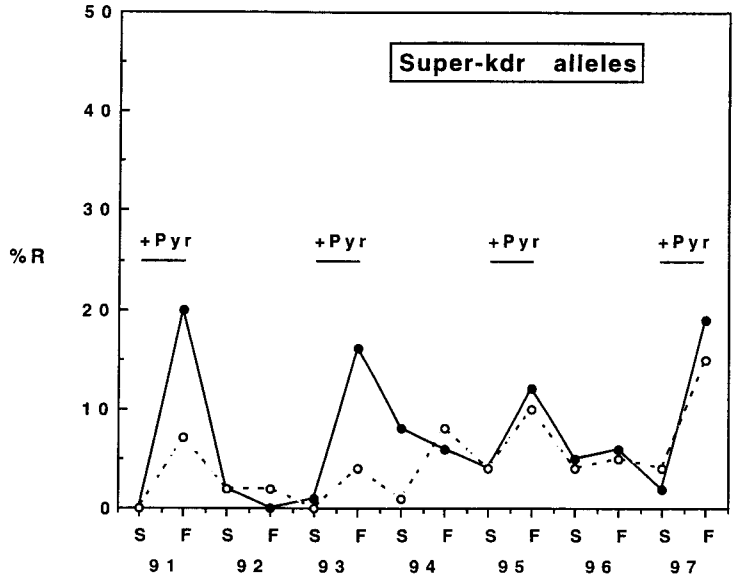


**Fig. 3.** Changes in pre- and posttreatment susceptible *superkdr* allele frequencies in male and female horn flies. Approximately 40 male and 40 female flies were genotyped from collections in the spring (S) before and in the fall (F) after ear tagging during 1991–1997. Percentage of R represents the percentage of sodium channel alleles in males (●) and females (○) that have the resistant *superkdr* genotype. Percentage of R = [(number of RR flies) × 2 + number of SR flies] ÷ 2 × total number of flies assayed] × 100%. The times during which the pyrethroid ear tags were in use are indicated (+Pyr).

sample, which had 71% SS *superkdr* female flies. Male *superkdr* RR flies were found only in three postpyrethroid treatments, the fall samples in 1991, 1993, and 1997 at levels of 4, 3, and 2%, respectively, and in one prepyrethroid treatment in 1995 at a level of 2%. The percentage of SS was slightly less prevalent in males than females, as four analyses of male samples contained <80% *superkdr* SS flies. These were each of the four postpyrethroid tag treatment fall samples in 1991, 1993, 1995, and 1997 which contained 63, 70, 76, and 65% *superkdr* SS male flies, respectively. The *superkdr* R allele frequency of both the males and females generally changed in concert with the exception of the 1993 prepyrethroid spring treatment and the 1994 postOP fall treatment samples (Fig. 3).

## Discussion

Insecticide resistance in the horn fly population from the St. Joseph's study site was quite high and resilient. During the 7-yr study, effective fly control was achieved only during the first year of OP ear tagging. Surprisingly, after having 15 wk of control in 1992 with OP ear tags, in 1994 the OP ear tag only controlled flies for 4 wk. Because pyrethroids were used in 1993, the lack of control in 1994 is likely due to a factor that had been genetically fixed in the population previously, was established and fixed during 1992, or was maintained in a resistance-induced status by the pyrethroid treatments. Because the target site of pyrethroids is the sodium channel while the target site of OPs is acetylcholinesterase (AChE), it was unlikely that the pyrethroid treatment of 1991 induced a resistance-causing genetic mutation in AChE. Although possible, but unlikely, an AChE mutation may have occurred near the end of the 1992 treatment that was stable in the local fly population until the spring of 1994 (while selection pressure by OP was nonexistent). This mutation could have been expressed to such an extent that in 1994 OP ear tags were not effective.

It is possible that a gene encoding an enzyme capable of detoxifying or sequestering both OP and pyrethroids was selected for early in the study and by 1994 had become genetically fixed to the extent that control failure by both classes of insecticide was the result. In 1993 and 1995, the resistance factor in the diazinon bioassays increased even though pyrethroid ear tags were used. This suggested the involvement of an enzyme active against both classes of insecticide. During the first 6 yr of the study, a pour-on synergized pyrethroid treatment was used. As discussed in Barros et al. (1999), this treatment, though effective for fly control in Louisiana for only ≈3–4 wk and recommended by the ear tag manufacturer, could have selected for individuals resistant to both OP and pyrethroids. The use of the yearly pour-on in our study was intended to aid in the selection of horn flies highly susceptible to the OP ear tags, as some pyrethroid resistant horn fly populations have been reported to possess negative cross-resistance with diazinon. Cilek et al. (1995) reported the likely involvement of the mixed function oxidase system in the diazinon negative cross-resistance found in pyrethroid resistant Kentucky horn fly populations. Our 1993 and 1995 bioassay data also indicated that OP negative cross-

resistance existed in the spring. However, by the fall collections in each of those two years after the pyrethroid taggings, both the $LD_{50}$ and resistance factors for diazinon had reached levels similar to those seen after the OP treatment of 1996. This suggested the pyrethroid treatment, possibly including the yearly pour-on, was responsible for activation of a metabolic factor also active against OP. Unfortunately, we cannot directly compare our results to those of Cilek et al. as they did not report what insecticidal pressures were affecting their fly populations at the time of sampling and their samples were taken at a single timepoint, while ours were taken before and after treatment. Nevertheless, in retrospect, the pour-on treatment should have been eliminated in the years of OP tagging, as the pour-on might be confounding a portion of the resting effect of the OP treatment on the distribution of pyrethroid susceptible alleles.

Target site insensitivity to pyrethroids has been reported to have a fitness cost in horn flies. Scott et al. (1997) reported that both pupation success and fecundity were negatively affected by target site pyrethroid resistance, resulting in a decrease of resistance in early season populations. There was an increase in percentage of S *kdr* alleles (and percentage of S *superkdr* alleles by implication, since *superkdr* R alleles are not found in the absence of a *kdr* R allele) in our study during the interval between the removal of pyrethroid ear tags in the fall and the resumption of pyrethroid pressure in the spring of the second year following (Fig. 2). Although we did not include fitness measures in our study, clearly, in both males and females, the susceptible alleles were selected for in the absence of pyrethroid pressure. Fitness costs were probably especially strong for *superkdr* resistance alleles. The percentage of R for *superkdr* alleles only rose above 10% after a season of pyrethroid ear tagging and rapidly fell once the pyrethroid ear tags were removed (Fig. 3). Lee et al. (1999) discussed studies of human and house fly sodium channel alleles which possess mutations corresponding to the *superkdr* M → T amino acid substitution and which have unusual metabolic responses to cold temperatures. It would be very interesting to determine if the selection against pyrethroid resistance *superkdr* alleles in the horn fly was due primarily to suboptimal response to low temperatures. This might be accomplished by using the *superkdr* PCR assay to genotype fly populations before and after diapause, a life stage during which low temperature fitness costs might be maximally expressed. Mwangala and Galloway (1993) reported that discontinuing the use of pyrethroid ear tags for a single season in Winnipeg, Canada did not substantially reduce the resistance levels. Thus, in conjunction with our study, skipping a season of pyrethroid ear tag use in both a hot and cold climate region in North America did not lead to reduced resistance levels.

Unfortunately, despite the increase in both the susceptible *kdr* and *superkdr* alleles in each interval between the use of pyrethroids, the proportion of homozygous susceptible flies never exceeded 19% and there was not a restoration of control by pyrethroids. Additionally, for the last two of the three no pyrethroid ("no Pyr") intervals of the study shown in Fig. 2, both the $LD_{50}$ and resistance factor decreased while the tag efficacy remained low (Table 2, 1993 post-1995 Pre- and 1995 post-1997 pre). Thus, in this study, neither $LD_{50}$, resistance factor nor percentage of S alleles was predictive of expected ear tag control efficacy. It appeared that despite the fitness costs associated with target site pyrethroid resistance, once the trait becomes fixed in the population, it is recalcitrant to short- or medium-term measures to restore susceptibility to pyrethroids.

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